Bacterial degradation of cyclohexane Participation of a co-oxidation reaction

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Complete biodegradation of cyclohexane was demonstrated to occur in a system containing two strains of Pseudomonad bacteria, viz an *n*-alkane oxidizer and a microorganism utilizing cyclohexanol. *n*-Heptane is also present as a substrate for the former. Neither bacterial strain was capable of utilizing cyclohexane individually.

This biodegradation of cyclohexane occurs in two steps:

(a) the conversion of cyclohexane into cyclohexanol by the *n*-alkane oxidizer (co-oxidation) and

(b) utilization of cyclohexanol by the second strain.

In unsuccessful attempts to accumulate a bacterium which could grow on cyclohexane as its sole source of carbon, we have used some uncommon methods, which are briefly described.

INTRODUCTION

There is an increasing interest in the microbial degradation of chemical compounds, e.g. hydrocarbons, in natural waters, in soil and in waste-water purification plants. Whereas the pathways of microbial degradation of a number of aliphatic and aromatic hydrocarbons are well known, little has been reported on the degradation of cycloparaffins.

We chose the simple cycloparaffin cyclohexane as a model for our studies on degradation pathways, because Imelik (1948), Colla and Treccani (1960), as well as Fredricks (1966) reported growth of a microorganism on cyclohexane. Because our attempts to isolate a microorganism capable of utilizing cyclo-

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hexane as the sole source of carbon and energy were not successful, we investigated the possibility of a mineralization of cyclohexane by co-oxidation or co-metabolism.

An indication for such a possibility was found in an earlier study (van Ravenswaay Claasen and van der Linden, 1971) where it was shown that intact Pseudomonas cells, induced to hydroxylate *n*-heptane, hydroxylated ethylcyclohexane at the 4-*trans* position. The cyclic alcohol thus formed accumulated in the medium.

Such a hydroxylation reaction might well be the first step in a co-oxidative mineralization process by a mixed culture of two different bacterial strains, each incapable of utilizing the parent hydrocarbon for growth.

MATERIALS AND METHODS

Culture medium. The composition of the mineral medium was the same as that described by van Ravenswaay Claasen and van der Linden (1971). The C-source is given in the text. Incubations were generally carried out in closed flasks containing (a) mineral medium, (b) an "oil phase" consisting of two (volatile) hydrocarbons and (c) a gas phase (air). A note on the concentration of the hydrocarbon compounds solubilized in the medium is given in the next paragraph.

Hydrocarbon concentration. In the three-phase system described above, the concentration in the medium of each of the component hydrocarbons can be calculated in principle by multiplying the solubility of the particular hydrocarbon in the medium with its mole fraction in the "oil phase".

It must be noted that the composition of the oil phase is not necessarily the same as that of the hydrocarbon mixture added. Because of evaporation, the (large) gas phase will be relatively richer in the more volatile hydrocarbon and the remaining oil phase correspondingly poorer. This again results in a lower concentration of the volatile component in the aqueous phase.

Selection of the n-*paraffin-utilizing microorganism.* Nine strains of *n*-alkane oxidizing Pseudomonads from the laboratory collection were tested for their capacity to hydroxylate cyclohexane (The method giving optimum results was developed in the course of this study and is mentioned below under "Accumulation of cyclohexanol through co-oxidation"). All strains were able to do so, but results shown in this report were obtained with one of these, tentatively identified as *Pseudomonas aeruginosa* (strain T) which was originally isolated from a contaminated kerosine sample.

Enrichment for and isolation of a cyclohexanol-utilizing bacterium. Since this

microorganism had to be used in a system containing cyclohexane, the isolate should not be affected adversely by this toxic hydrocarbon. Therefore, the following procedure was followed: A mixed culture of cyclohexanol-utilizing bacteria was obtained by enrichment from harbour water in 50 ml mineral medium with 0.02% cyclohexanol in closed 500 ml flasks. By gradually increasing the cyclohexanol concentration from one subculture to the next at last good growth was obtained at a concentration of 0.2% cyclohexanol. We then adapted this mixed culture to increasing concentrations of cyclohexane. At first even minute amounts of about 5 µl per flask decreased the growth rate considerably, but after subculturing for several weeks in the presence of increasing amounts (up to 100 µl per flask) of cyclohexane all but one species were suppressed and the surviving one could be isolated by plating on 0.2% cyclohexanol mineral agar. The microorganism, probably a *Pseudomonas* species, was subcultured on 0.2% cyclohexanol mineral agar slants.

Attempts to isolate a cyclohexane-utilizing organism. In spite of strenuous efforts no bacterium able to utilize cyclohexane as the sole carbon source could be isolated. The techniques used in these unsuccessful attempts are documented in this section. In particular the volatile hydrocarbons of low molecular weight and with a relatively high solubility in water, such as benzene and pentane, are toxic to most microorganisms. However, microorganisms capable of growth on such hydrocarbons may be isolated from enrichment cultures in which these hydrocarbons are present at sub-saturated concentrations. In order not to limit the amount of hydrocarbon present in the medium to impractically low values the sub-saturated concentrations are preferably based on some equilibrium. The three methods tried were:

(1) Aliquots of mineral medium from 5 up to 50 ml, previously saturated with cyclohexane in a separating funnel, were introduced in 500-ml closed flasks and left standing for one hour for equilibration of cyclohexane between air and water phase before inoculation.

(2) In a series of stoppered flasks containing mineral medium, small test tubes were placed containing an *iso*octane-cyclohexane mixture, the mixing ratio increasing from 1:1 to 10:1 (v/v). *Iso*octane is a non-degradable hydrocarbon. It is of low toxicity and serves to keep the cyclohexane concentration far below saturation. (See note on hydrocarbon concentration above.)

(3) Mineral medium (50 ml) plus 10 µl cyclohexane was placed in closed flasks of 500 ml and from 10 µl to 100 µl of non-utilizable *iso*octane were added on top of the medium. The difference with (2) is in the lower amount of hydrocarbons introduced and in its direct contact with the culture.

The inocula used were obtained from oil-containing effluents and soil samples obtained at a refinery. Though we found it easy to isolate e.g. benzene-utilizing

microorganisms with either one of these techniques, our efforts to isolate a cyclohexane-utilizing bacterium remained unsuccessful.

Accumulation of cyclohexanol through co-oxidation. For the demonstration of the formation of cyclohexanol from cyclohexane the following conditions were chosen. The *n*-alkane oxidizer was grown in 500 ml glass-stoppered Erlenmeyer flasks in a culture volume of 50 ml with 0.3 ml *n*-heptane. After incubation for 16 hr at 30 C in a shaker bath the cells – usually grown to an optical density (O.D.) of approximately 1.0 – were harvested and washed twice with 0.06 M phosphate buffer of pH 7.4. After dilution with phosphate buffer to an O.D. of 0.4 (240 μg dry wt cells per ml) the bacterial suspension (5 ml) was placed in 50 ml glass-stoppered Erlenmeyer flasks. For optimum results 30 μl of a 2:1 (v/v) mixture of heptane and cyclohexane was added to the flasks. After incubation at 30 C in a shaker bath for 18 hr the contents were analysed for the formation of cyclohexanol by Gas Liquid Chromatography (GLC) on a Porapak Q column (Waters Associates, Inc.). GLC analysis on a 6% DEGS column (Diethyleneglycol succinate on Chromosorb W support, Hewlett Packard) was used to ascertain that indeed cyclohexanol was formed and no cyclohexanone was present.

Co-oxidative degradation of cyclohexane. When a system was used containing two bacterial strains the conditions were as follows: the bacteria were suspended in a total of 50 ml mineral medium and 150 μl of a 2:1 mixture of heptane and cyclohexane was added. No liquid hydrocarbon is present in this system. The concentration of cyclohexane in the medium was approximately 6–7 μg/ml or about 12% of its saturation value.

The 500 ml flasks also contained a test tube with 2 ml of a 10% solution of NaOH in water when ^{14}C-cyclohexane was used and $^{14}CO_2$ had to be measured. The closed flasks were incubated at 30 C for 18 hr before analysis by GLC and determination of the radioactivity.

Analysis for ^{14}C radioactivity. The experiment described in the preceding paragraph in which we used ^{14}C-cyclohexane, was worked up as follows:

(a) Carbon dioxide. The contents of the test tube with NaOH were transferred quantitatively to a counting vial, stripped three times with 5 ml portions of cyclohexane at 85 C to remove any dissolved ^{14}C-cyclohexane left, and counted (see below).

(b) Bacterial cells. Ten ml of the culture fluid was centrifuged. The cells were washed once with phosphate buffer and transferred to a counting vial, stripped with cyclohexane as described above, and counted. The counts obtained were recalculated to represent the total amount of radioactivity of the cells contained in the flask.

(c) Culture liquid. The supernatant liquid obtained by centrifugation was

analysed quantitatively for the presence of cyclohexanol by GLC as described above. Stripping the liquid with cyclohexane, as described in the analysis of labelled carbon dioxide and bacterial cells, resulted in a large loss of cyclohexanol. Therefore the quantified amount of cyclohexanol was converted into disintegrations per min by using the specific activity of the cyclohexane from which it was formed (6.4×10^4 dpm/mg).

To ascertain that no radioactive carbon dioxide had remained in the culture liquid and that no acidic metabolites had been formed, 2 ml of the supernatant liquid was made alkaline and was counted after extraction with cyclohexane. No significant radioactivity was found (<100 dpm), as calculated for the total contents of the flask.

As all samples were aqueous liquids the contents of the counting vials were made up to a volume of 4 ml with water before addition of 12 ml of Instagel (Packard, Brussels), a scintillation emulsifier on the basis of toluene-xylene. A Packard model 3003 scintillation counter was used for counting the gels.

RESULTS

Before starting our detailed studies we first ascertained that the co-oxidative properties of the *n*-heptane-grown *Pseudomonas* 473, as described by van Ravenswaay Claasen and van der Linden (1971) (See Introduction) are not restricted to this particular strain. To do this, we carried eight other *Pseudomonas* strains through a procedure similar to the optimized procedure now described under "Methods". We found that in every case resting cell suspensions of *n*-heptane-grown cells converted cyclohexane into cyclohexanol. From this result we concluded that this co-oxidation reaction is not a rare phenomenon and that it would be worthwhile to demonstrate its possible contribution to the mineralization process unequivocally.

Early experiments with growing cells showed that upon addition of cyclohexane to bacteria actually growing on *n*-heptane the culture ceased to grow while little or no cyclohexanol was formed, probably because of a competition between the two substrates for one enzyme: the paraffin ω-hydroxylase. It was clear that the conditions for the formation of cyclohexanol by cells actually growing on *n*-heptane had to be optimized.

Fig. 1 shows the accumulation of cyclohexanol in the presence of constant amounts of *n*-heptane and variable amounts of cyclohexane. At lower concentrations of cyclohexane the formation of cyclohexanol increases with increasing concentrations of cyclohexane, but a maximum is reached at a ratio of 2:1 (v/v) heptane-cyclohexane. The decrease at higher concentrations is due to the toxi-

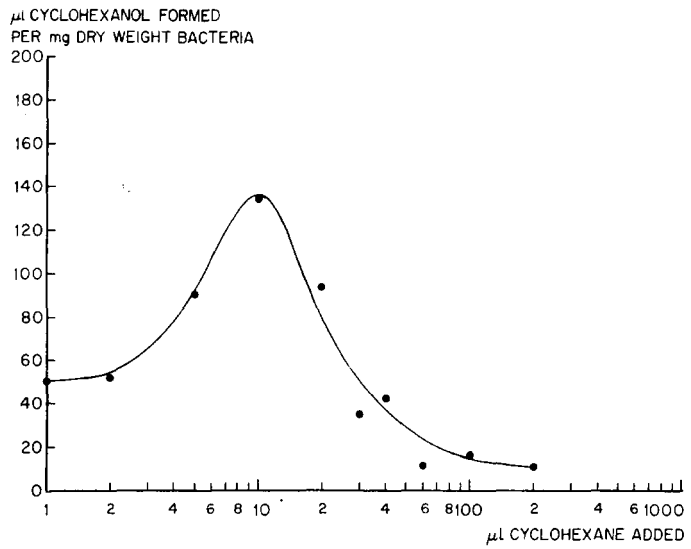

Fig. 1. Formation of cyclohexanol by *Pseudomonas* T at different concentrations of cyclohexane.
Twenty µl *n*-heptane present in all 50 ml flasks containing 5 ml bacterial suspension. For further details see: Materials and Methods.

city of cyclohexane. For this reason we also tested the effect of increasing amounts of the optimum 2:1 heptane-cyclohexane mixture in the same system.

Fig. 2 shows that again a maximum is reached. Quite coincidentally this maximum was found at approximately the same total hydrocarbon concentration as used in the first series of experiments (compare with Fig. 1).

It should be emphasized here that under the conditions applied the amounts of hydrocarbon used are normal substrate quantities, customary for the growth of bacteria (0.1–0.5%). Owing to the low solubility and the high volatility of the hydrocarbons used, they are mainly present in the gas phase and as a drop of liquid floating on top of the culture.

It has been explained under "Hydrocarbon concentration" (see: Materials and Methods) why actual concentrations in the medium are not easily calculated in the three-phase system, but in all experiments the concentration of cyclohexane in the medium was below its saturation value.

As expected, we found that upon addition of the cyclohexanol-utilizing microorganism 50% of the cyclohexanol formed in the experiment was consumed in about one hour. After 18 hr the oxidation of cyclohexanol appeared to be completed.

The above experiments tended to confirm our views about a possible co-

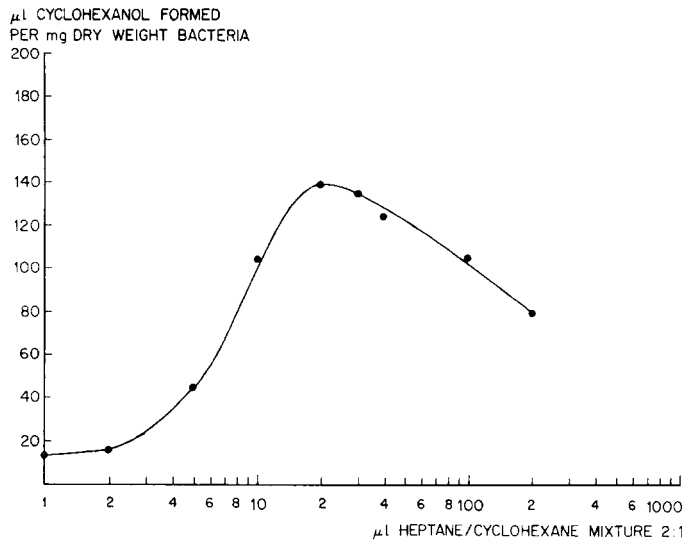

Fig. 2. Formation of cyclohexanol by *Pseudonomas* T at different amounts of a 2:1 mixture of *n*-heptane and cyclohexane in 50 ml flasks containing 5 ml bacterial suspension. For further details see: Materials and Methods.

oxidative mineralization of cyclohexane, but it remained to be shown for the complete system containing two bacterial strains and two hydrocarbons:

(a) that the *n*-alkane oxidizer cannot further degrade the cyclohexanol formed,

(b) that the cyclohexanol-utilizing bacterium oxidizes cyclohexanol completely, converting it into carbon dioxide, water and newly formed cell material exclusively and

(c) that degradation of cyclohexane by the cyclohexanol-oxidizer does not occur.

To demonstrate this we used ^{14}C-labelled cyclohexane and performed an analysis for the presence of radioactivity in carbon dioxide, water and bacterial cells. The experiment was set up on a larger scale. (See: Materials and Methods.)

The results summarized in Table 1 show that in flask I, containing only the *n*-alkane oxidizer, no radioactivity was found in the cells or in the carbon dioxide. Evidently, the ^{14}C-cyclohexanol formed was not utilized by the *n*-alkane oxidizer.

The contents of flask II, designed for further degradation of the cyclohexanol formed, showed upon analysis – as expected – radioactivity to be present in carbon dioxide and in cells as a result of ^{14}C-cyclohexanol-utilization. The oxidation of ^{14}C-cyclohexanol was complete: no cyclohexanol could be detected

Table 1. Co-oxidative degradation of ^{14}C-cyclohexane in a system with two *Pseudomonas* strains

Composition of the system				
Flask[1]	*Ps.*T O.D.1.6	*Ps.*"cyclohexanol" O.D.2.5	^{14}C-cyclohexane	Heptane
I	5 ml	–	50 µl	100 µl
II	5 ml	5 ml	50 µl	100 µl
III	–	5 ml	50 µl	100 µl
Results				
Flask	$^{14}CO_2$ dpm	^{14}C-cyclohexanol GLC µg	^{14}C-cyclohexanol dpm calculated[2]	^{14}C-cells dpm
I	86	198	10496	118
II	7580	<1	<100	6039
III	103	<1	<100	41

[1] The 500 ml flasks contained enough mineral medium to make up a total volume of 50 ml bacterial suspension.
[2] The radioactivity of cyclohexanol was calculated from the GLC determination for reasons described under "Materials and Methods".

by GLC (see this table) and no acidic products derived from the labelled cyclohexanol could be detected in the medium (see Materials and Methods).

In flask III the absence of radioactivity in carbon dioxide and cells is proof that the cyclohexanol oxidizer does not utilize ^{14}C-cyclohexane.

Admittedly the formation of 198 µg of cyclohexanol from 50 µl or 35 mg cyclohexane in flask I corresponds to a conversion of as little as 0.56%. However, this low figure is due to the fact that in order to identify and quantify the amount of ^{14}C-cyclohexanol formed, the optimum conditions for its maximum formation were used. Conditions for an optimum conversion, however, are quite different.

Nevertheless Figs. 1 and 2 already show that conversion is better at lower hydrocarbon concentrations.

DISCUSSION AND CONCLUSION

Foster (1962) proposed the term co-oxidation to describe incomplete oxidation of a compound by a microorganism which is not able to utilize that compound for growth. Horvath and Alexander (1970) hold this co-oxidation (or co-metabolic) mechanism responsible for the incompleteness of the degradation

of some pesticides. Our study, however, concerns a co-oxidative reaction accomplished by one microorganism, which initiates complete degradation by a second bacterium.

We demonstrated that the degradation of cyclohexane is effected by consecutive action of two *Pseudomonas* strains in the presence of *n*-heptane; the *n*-alkane oxidizer co-oxidizes cyclohexane to cyclohexanol and this product is utilized by the other Pseudomonad.

We do not believe co-oxidative hydroxylation of cyclohexane to be a strange or rare phenomenon. It is merely the consequence of the non-specificity of the alkane hydroxylase in the alkane oxidizer. This enzyme can also accommodate hydrocarbons other than *n*-alkanes. Which hydrocarbons can be accommodated and, hence, co-oxidized was shown to depend on the geometric configuration of the active centre of the enzyme and on the dimensions of the co-substrate molecule (van Ravenswaay Claasen and van der Linden, 1971).

We have tried to isolate a bacterium utilizing cyclohexane as its only source of carbon, but without success. Of course this does not mean that such a bacterium does not exist. We did show, however, that co-oxidation – which may be called a metabolic mishap caused by the non-specificity of an enzyme – can make an otherwise not degradable compound amenable to further degradation.

There is no reason to believe that co-oxidative or co-metabolic phenomena are restricted to hydrocarbons or to hydroxylating reactions.

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REFERENCES

COLLA, C. e TRECCANI, V. 1960. Metabolismo ossidativo microbico degli indrocarburi ciclici saturi.—Ann. Microbiol. Enzimol. **10**: 77–81.

FOSTER, J. W. 1962. Bacterial oxidation of hydrocarbons, p. 241–271. *In* O. Hayaishi, (ed.), Oxygenases.—Academic Press, Inc., New York.

FREDRICKS, K. M. 1966. Adaptation of bacteria from one type of hydrocarbon to another.—Nature (London) **209**: 1047–1048.

HORVATH, R. S. and ALEXANDER, M. 1970. Cometabolism of *m*-chlorobenzoate by an *Arthrobacter*.—Appl. Microbiol. **20**: 254–258.

IMELIK, B. 1948. Oxydation du cyclohexane par *Pseudomonas aeruginosa*.—C. R. Acad. Sci. **226**: 2082–2083.

VAN RAVENSWAAY CLAASEN, J. C. and VAN DER LINDEN, A. C. 1971. Substrate specificity of the paraffin hydroxylase of *Pseudomonas aeruginosa*.—Antonie van Leeuwenhoek **37**: 339–352.